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CLAIMS

1. A method for identifying a modulator in the form of a biologically active polypeptide fragment which is capable of detectably modulating, *in vivo*, a phenotypic trait in a cell, the method comprising the steps of

(a) preparing a pool of expression vectors, each vector of said pool containing at least one member from a library of randomly modified nucleotide sequences derived from a parent nucleotide sequence encoding a parent peptide which *in vivo* directly modulates activity of a known protease, wherein the randomly modified nucleotide sequences comprise

- an invariable part encoding a scaffold portion of the parent peptide, said scaffold portion serving to stabilize said polypeptide fragment and being stable towards proteolytic attack and/or being insensitive to a reducing environment, and
- random nucleotides,

(b) transforming a population of substantially identical cells with said vectors of said pool so as to obtain transformed cells,

(c) culturing said transformed cells under conditions facilitating expression of said randomly modified nucleotide sequences,

(d) examining said transformed cells and isolating transformed cell(s) wherein the preselected phenotypic trait is modulated thereby indicating that the expression product of said randomly modified nucleotide sequence is biologically active, and

(e) identifying the modulator by determining said randomly modified nucleotide sequence of said vector present in cell(s) isolated in step (d) and/or determining the amino acid sequence of the expression product encoded by said randomly modified nucleotide sequence.

2. The method according to claim 1, wherein the substantially identical cells are prokaryotic cells.

3. The method according to claim 1, wherein the substantially identical cells are eukaryotic cells.

4. The method according to claim 3, wherein the eukaryotic cells are selected from the group consisting of fungal cells, protozoan cells, animal cells, and plant cells.

5. The method according to claim 4, wherein the animal cells are selected from the group consisting of mammalian cells, arthropod cells such as insect cells, avian cells, and piscine cells.

6. The method according to any of the preceding claims, wherein the transformed cells examined in step (d) predominantly carries one single copy of the vector.

7. The method according to claim 6, wherein transformation step (b) is performed under such conditions that the cells transformed are predominantly or at most transformed with one single vector from said pool, or wherein, prior to carrying out step (d), cells being transformed with more than one vector from said pool are substantially excluded from the further steps.

8. The method according to any one of the preceding claims, wherein the random nucleotides are introduced in part(s) of the parent nucleotide sequence which encode(s) the active site(s) of the parent peptide, or the part(s) which encode(s) structure(s) interfering with the active site(s).

9. The method according to any one of the preceding claims, wherein the invariable part of the nucleotide sequence encodes truncated parts of the scaffold portion of the parent peptide sufficient to maintain stability.

10. The method according to any of the preceding claims, wherein the invariable part of the parent nucleotide sequence encodes a peptide which is free from disulfide bridges.

11. The method according to any one of claims 1-9, wherein the invariable part of the parent nucleotide sequence encodes a peptide having disulfide bridges.

12. The method according to any one of the preceding claims, wherein the random nucleotides are introduced in the form of an insertion or a substitution into the parent nucleotide sequence, optionally in combination with deletion(s) in the parent nucleotide sequence.

13. The method according to claim 12, wherein the number of random nucleotides which are introduced is in the range from 3 to about 100.

14. The method according to any one of the preceding claims, wherein the random nucleotides are nucleotide sequences and/or are single random nucleotides introduced at specific sites in the parent nucleotide sequence.

15. The method according to any one of the preceding claims, wherein the random nucleotides are selected from the group consisting of

- synthetic, completely random deoxyribonucleotides;
- synthetic random DNA sequences, wherein limitation on randomization of some nucleotides is introduced so as to limit the number of available sequences and/or to avoid undesired stop codons and/or to facilitate introduction of post-translational modifications of expressed peptide(s);
- synthetic random DNA sequences as in (1) or (2) coupled to a sequence encoding a purification tag; and
- CDR encoding nucleotide sequences isolated from a library of immune-competent cells raised against an antigen.

16. The method according to claim 15, wherein the CDR encoding nucleotide sequences encode CDR-3 peptide sequences.

17. The method according to any one of claims 14-16, wherein the random nucleotides are prepared by random codon synthesis where defined DNA codons are synthesized in a random order.

18. The method according to claim 17, wherein the relative amount of synthesized codons ensure that all encoded amino acids will be present with substantially the same frequency in the total of encoded polypeptide fragments.

19. The method according to any one of the preceding claims, wherein the random nucleotides are introduced into the expression vector by the principle of site directed PCR-mediated mutagenesis.

20. The method according to any one of the preceding claims, wherein the parent peptide is an inhibitor of activity of the known protease.

21. A method according to claim 20, wherein the inhibitor is selected from the group consisting of a BPTI/Kunitz family protease inhibitor, a serpin family protease inhibitor, a Kazal family protease inhibitor, a soybean trypsin inhibitor (Kunitz) family protease inhibitor, a potato inhibitor I family member, a Bowman-Birk family protease inhibitor, a squash inhibitor family member, a wap-type 'Four-disulfide Core' proteinase inhibitor, a hirudin family protease inhibitor, a factor Xa inhibitor, an Ascaris trypsin inhibitor family member, a cystatin family protease inhibitor, a calpain family cysteine protease inhibitor, a tissue inhibitor of metalloproteinases family member, a carboxypeptidase A inhibitor, a metallocarboxypeptidase inhibitor, and an angiotensin-converting enzyme inhibitor.

22. The method according to claim 21, wherein the parent peptide is a potato inhibitor family I member.

23. The method according to claim 22, wherein the parent peptide is chymotrypsin inhibitor 2A (CI-2A).

24. The method according to ~~any of the preceding claims~~ wherein the substantially identical cells are mammalian cells and the vector is selected from the group consisting of a retroviral vector, a vaccinia virus vector, an adenoviral vector, an adeno associated virus (AAV) vector, a herpes simplex virus (HSV) vector, an alpha virus vector, and a semliki forest virus vector.

25. The method according to claim 24, wherein the vector is retroviral.

26. The method according to claim 25, wherein the retroviral vector is derived from retrovirus selected from the group consisting of Avian Leukosis-Sarcoma Virus (ALSV), Mammalian type C, Mammalian type B, and Lentivirus, and optionally modified with heterologous cis-acting elements.

27. The method according to claim 25 or 26, wherein the retroviral vector has non-identical ends.

28. The method according to claim 27, wherein the non-identical ends contain non-identical promoters.

29. The method according to ~~any one of~~ claims 25-~~28~~, wherein the retroviral vector contains a heterologous promoter replacing the viral promoter in the 5'-LTR, such as a CMV promoter, an RSV promoter, an SV-40 promoter, a TK promoter, an MT promoter, or an inducible system such as Tet or Ecdysone.

30. The method according to ~~any one of~~ claims 25-~~29~~, wherein step (a) is carried out by

1) transfecting suitable packaging cells with vectors which comprise the randomly modified nucleotide sequences and which are integratable in virions produced by said packaging cells,
2) culturing said transfected packaging cells in a culture medium under conditions which facilitate production by the packaging cells of virions containing the randomly modified nucleotide sequences,

3) recovering and optionally concentrating said virions, and

4) transducing said substantially identical cells with the virions.

31. The method according to claim 30, wherein the packaging cells are selected from the group consisting of PE501, Bosc23, Ψ2, GP+E86, PhoenixEco, PA317, GP+AM12, DA(ampho), Bing, FLYA13, ProPak, CRIP, ΨAM, Phoenix-Ampho, PG13, H9 (293GPG), and EcoPack.

32. The method according to ~~any one of~~ claims 25-31, wherein the virions are pseudotyped retrovirus produced by an ecotropic packaging cell line so as to confer broad tropism to the virions produced thereby, or wherein an ecotropic receptor has been introduced into the substantially identical cells so as to allow transduction with ecotropic virions.

33. The method according to claim 32, wherein the ecotropic receptor has been introduced in the substantially identical cells by means of transduction.

34. The method according to ~~any of the preceding~~ claims wherein the randomly modified nucleotide sequences are coupled to a nucleotide sequence encoding at least one fusion partner.

35. The method according to claim 34, wherein the fusion partner serves to facilitate expression and/or purification/isolation and/or further stabilization of the expression product.

36. The method according to claim 35, wherein the fusion partner includes a purification tag such as His_6 tag, myc tag,

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BSP biotinylation target sequence, of BirA, flu tag, lacZ, and GST.

37. The method according to claim 34 or 35, wherein the fusion partner is a sorting signal or a targeting sequence.

38. The method according to claim 37, wherein the sorting signal is a signal patch or a signal peptide.

39. The method according to claim 37 ~~or 38,~~ wherein the sorting signal effects export of the expressed peptide out of the cell or into the cell membrane, or, when the substantially identical cells are eukaryotic, into endoplasmic reticulum, into Golgi apparatus, into lysosomes, into secretory vesicles, into mitochondria, into peroxisomes, or into the nucleus.

40. The method according to ~~any one of the preceding claims~~, which further comprises the step of resolving the 3-dimensional structure of the identified modulator.

41. A method for the preparation of a replicable expression vector, the method comprising the steps of identifying a modulator by the method according to ~~any one of the preceding~~ claim~~s~~, and subsequently

i) isolating or synthesizing a nucleic acid sequence which encodes the modulator, and

ii) engineering a replicable expression vector comprising an operon which comprises, in the 5'-3' direction and in operable linkage, 1) a promoter for driving expression of the nucleic acid sequence, 2) optionally a nucleotide sequence encoding a leader peptide, 3) the

nucleic acid sequence, and 4) optionally a termination signal.

42. A method for the preparation of a transformed cell carrying a nucleic acid sequence encoding a modulator as defined in any one of claims 1-40, the method comprising transforming a suitable host cell with an expression vector prepared according to claim 41.

43. A method for providing a modulator as defined in any one of claims 1-40, the method comprising

I) growing a transformed cell prepared according to the method of claim 42 in a culture medium under conditions which facilitates expression by the cell of the randomly modified nucleotide sequence, and

II) subsequently harvesting the expression product from the cell and/or the culture medium, or

Ia) identifying the modulator according to the method of ~~any one of~~ claims 1-~~40~~, and

Ib) subsequently synthesizing the modulator by means of chemical synthesis on the basis of the sequence determined in step (e).

44. A method for isolating and/or identifying a target biomolecule, the method comprising providing a modulator according to the method of claim 43 and subsequently using the modulator as an affinity ligand in an affinity purification step so as to isolate the target biomolecule from a suitable sample.

45. The method according to claim 44, wherein the affinity purification step is an affinity chromatographic step, an

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affinity mass spectrometry step, or a co-immunoprecipitation step.

46. A method for isolating and/or identifying a target biomolecule, the method comprising providing a peptide modulator according to the method of claim 42 and subsequently using the modulator as a probe against a cDNA library derived from the substantially identical cells or using the modulator as bait in a two- or three-hybrid system.

47. The method according to any of claims44-46, wherein the target biomolecule is a peptide or a nucleic acid.

48. The method according to claim 47 further comprising the step of determining the amino acid sequence of the peptide or determining the nucleotide sequence of the nucleic acid.

49. The method according to ~~any of~~ claim~~s~~ 44~~-48~~, further comprising the step of resolving the 3-dimensional structure of the target biomolecule.

50. A method for selecting a chemical compound as a putative drug candidate in drug development, the method comprising the steps of

- assaying a library of chemical compounds for interaction with a target biomolecule which has been de novo isolated according to the method of ~~any one of~~ claim~~s~~ 44~~-49~~, and
- selecting compounds which interact significantly with the target biomolecule.

51. The method according to claim 50, wherein the library of chemical compounds has been provided by chemical synthesis

upon initial identification of the members of the library by structure-based or non-structure based computer drug-modelling.

52. A method for the preparation of a medicinal product, the method comprising the steps of

A) selecting a chemical compound by the method according to claim 50 or 51,

B) performing pre-clinical tests with the chemical compound in order to assess the suitability thereof as a medicinal product,

C) entering, if the chemical compound is deemed suitable in step (B), clinical trials using the chemical compound in order to obtain market authorization for a medicinal product including the lead compound as a pharmaceutically active substance, and

D) upon grant of a market authorization, admixing the chemical compound with a pharmaceutically acceptable carrier, excipient or diluent and marketing the thus obtained medicinal product.

53. A method for developing a medicinal product, the method comprising that a modulator identified according to the method of ~~any one of~~ claims 1-~~40~~ serves as a lead compound in the drug development phase or wherein a target biomolecule isolated/identified according to ~~any one of~~ claims 44-~~49~~ serves as an interaction probe for the identification of putative drug candidates in the drug discovery phase.